JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v3i2.140

Proteomic changes in an extended-spectrum beta-lactamase-producing *Escherichia coli* strain under cefotaxime selection

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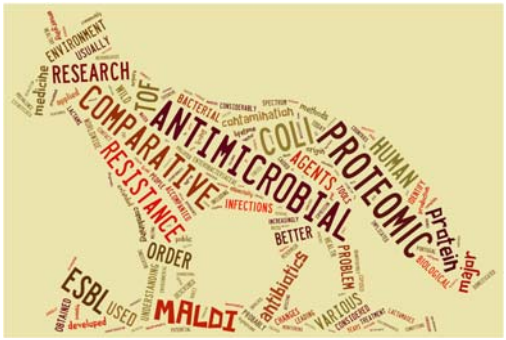
Received: 15 May 2013 **Accepted:** 18 September 2013 **Available Online**: 09 October 2013

Abstract

Proteomics can be used to study the metabolic pathways and mechanisms involved in antimicrobial resistance. The aim of this comparative proteomic study was to establish the overall changes in the proteome of a naturally occurring ESBL-producing *E. coli* strain (C5478) stressed with its minimal inhibitory concentration (2 µg/mL) of cefotaxime, compared to the proteome of the same strain without antimicrobial stress, by using 2-D gel electrophoresis (2-DE) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The comparative proteomic analysis revealed that the abundance of numerous protein species changed in the strain stressed by CTX compared to the non-stressed wild-type strain. A total of 188 spots were excised from the 2-DE gel of the wild-type strain, 112 of which were successfully identified by MALDI-TOF MS, representing 110 different proteins. Concerning the 2-DE gel of the CTX-stressed bacteria, 171 spots were excised and 156 were identified, representing 143 different proteins. The proteins identified in both strains were categorized according to their biological functions. These proteins were involved in metabolism, protein synthesis, cell division, stress responses, and antimicrobial resistance, among others. These findings will be helpful to further understand not only the antimicrobial resistance mechanisms, but also the role of wild animals as reservoirs in spreading antimicrobial-resistant bacteria into the environment.



Keywords: Proteomics; Cefotaxime; Antimicrobial resistance; *Escherichia coli*; MALDI-TOF-MS.

1. Introduction

Cefotaxime is one of the most widely used parenteral third generation cephalosporins in medicine [1]. However, shortly after the introduction of the first β-lactam antibiotics, some bacteria were found to be resistant, comprising the long-term usefulness of these drugs [2]. A common resistance mechanism is the expression of β-lactamase enzymes by bacteria, which inactivate the antibiotics by cleaving the β-lactam core. Resistant extended-spectrum β-lactamases (ESBL)-producing bacteria are thus a major clinical and public health concern in various countries, including Portugal, as they are commonly implicated in human infections [3].

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The appearance of multiresistant bacterial strains in humans or animals in clinical situations can also lead to contamination of the environment [4]. The production of ESBLs by *Enterobacteriaceae*, and especially *Escherichia coli*, has been repeatedly described in apparently healthy animals in the last few years [5]. Wildlife is not usually exposed to antimicrobial agents in clinical use but may acquire antimicrobial-resistant bacteria through contact with humans, domesticated animals and the environment. Water polluted with faeces is probably the most significant route of contamination. Monitoring the prevalence of resistance in indicator bacteria such as *E. coli* in wild animals could show whether wildlife serves as an environmental reservoir or "melting pot" of bacterial resistance.

Proteomics techniques can be used to quantitatively compare the expression of protein in different conditions to obtain a better understanding of the cellular processes affecting their expression and/or in which they are involved. Mostly two-dimensional electrophoresis (2-DE) is used for extensive protein separation before identification. Mass spectrometry (MS) in combination with various protein resolution methods and bioinformatics tools is used routinely in proteomics research [6]. For example, 2-DE and MALDI-TOF MS have been used several times to identify antimicrobial-related proteins in *E. coli* strains [7-10].

The scientific community is developing several tools in order to address the problem of antimicrobial resistance [11, 12]. Proteomics research offers major opportunities to characterize bacterial pathogens, elucidate disease mechanisms and to identify new diagnostic markers and therapeutic targets [13]. Comparative proteomic analyses of different bacterial species have shown that a subproteome related to resistance mechanisms changes when some strains are challenged with antimicrobials, e.g., in *E. coli* isolates treated with piperacillin/tazobactam [14], nalidixic acid [15], tetracycline [16, 17], streptomycin [18], chloramphenicol [19] and ampicillin [17]; in *Salmonella* treated with fluoroquinolones [20]; in *Stenotrophomonas maltophilia* treated with imipenem [21]; and in *Acinetobacter baumannii* treated with tetracycline and imipenem [22].

To better understand the universal pathways that form barriers to antimicrobial agents, the aim of this comparative proteomic study was to establish the overall changes between a wild-type strain (ESBL-producing *E. coli* strain C5478) and the same strain stressed with cefotaxime.

2. Material and methods

2.1. *Isolation of bacteria from fox faeces*

The *E. coli* strain C5478, referred to as the wild-type strain, was characterized in a previous study [23]. Red fox faecal samples were collected from February-2008 to March-2009 in North of Portugal (where these animals live in the wild) during hunts of red foxes. This kind of hunting is organized all of the years during a short period of time having like main objective the ecological control of the animal population and is supervised by the Agriculture, Rural development and Fishery Ministry of Portugal under the Decree-Law no. 202/2004. The animals were obtained in collaboration with National Corporation of Forest Rangers. As far as we know, none of the animals had received antimicrobials. Faecal samples were seeded into Levine agar supplemented with CTX (2 μg/mL).

2.2. *Genetic characterization of E. coli C5478*

C5478 showed resistance to ampicillin, amoxicillin + clavulanic acid, CTX, ceftazidime, aztreonam, tetracycline, sulfamethoxazole/trimethoprim, streptomycin, nalidixic acid, ciprofloxacin and chloramphenicol. The β-lactamase genes detected in C5478 are $bla_{\text{SHV-12}} + bla_{\text{TEM-1b}}$. C5478 also harbours the genes *tet*(A) and *sul*2 and contained the non-classical class 1 integron (*intI*1-*dfr*A12-*orf*F-*aad*A2-*cml*A1-*aad*A1-*qacH*-IS*440*-*sul*3) with the PcH1 promoter. The presence of two amino acid changes in GyrA (S83L and D87G) and one in ParC (S80I) were responsible for the ciprofloxacin and nalidixic acid resistance phenotypes detected in C5478. This isolate was typed as sequence type ST1086, phylogroup A and carried the *fim*A virulence gene [23].

2.3. *Culture conditions and total protein extraction*

The "stressed strain", was obtained by culturing the "wild-type strain" (ESBL-producing *E. coli* strain C5478) into brain heart infusion (BHI) broth containing 2 μg/mL CTX (Oxoid, Cambridge, UK). For this study, *Escherichia coli* strain K-12 was used as the negative control. In the post-exponential phase (when the optical density of the culture OD_{540nm} = 6 corresponding to 2–3 × 10^9 cells/mL), the cells were pelleted at 10,000 rpm at 4ºC for 3 min. The pellet was resuspended in an equal volume of pre-warmed phosphate-buffered saline (PBS) pH 7.4 [24]. After a second centrifugation, the pellet was resuspended in 0.2 mL of SDS sample solubilization buffer. The sample was sonicated with an ultrasonic homogenizer (three 10-s bursts at 100 W and 4 °C). The disrupted cells were centrifuged in an Eppendorf microfuge at maximum speed (14,000 *g*) for 30 minutes at 4°C. For SDS-PAGE experiments the supernatant was collected and resuspended in an equal volume of buffer containing 0.5 M Tris HCl pH 8.0, glycerol, SDS and bromophenol blue.

2.4. *SDS-PAGE and staining*

SDS-PAGE was performed on vertical gels (12.5%T and 0.97%C) in a Hoefer™ SE 600 Ruby® (Amersham Biosciences) unit, according to the procedure described by Laemmli [25] with some modifications. Electrophoresis was carried out at constant amperage of 30 mA per gel until the dye-front reached the bottom of the gels that were then stained with Coomassie Brilliant Blue R250 and washed in water overnight. Gels were then fixed in 6% trichloroacetic acid for

4 h and in 5% glycerol for 2 h [26].

2.5. *Two-dimensional electrophoresis and proteomics analysis*

Immobiline™ pH Gradient (IPG) technology (Görg et al., 2007) was used for 2-DE [27]. Protein samples from the wild -type strain were compared to protein samples from the stressed strain by subculture at the minimal inhibitory concentration of CTX. For isoelectric focusing (IEF), precast 13-cm IPG strips with linear gradient of pH 3-10 (pH 3–10 NL, Amersham Biosciences, UK) were passively rehydrated overnight in a reswelling tray with rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte IPG buffer pH 3-10) at room temperature. IPG strips were covered with DryStrip Cover Fluid (Plus One, Amersham Biosciences). Lysis buffer (9.5 M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3-10) and 10 mM Pefabloc® proteinase inhibitor) was added to the bacteria (1:1, v/v). Samples containing a total of 100 µg of protein were cup loaded on to the rehydrated IPG strips [28] and proteins were focused sequentially at 500 V for 1 h, 1000 V for 8 h, 8000 V for 3 h and finally 8000 V for 1 h 30 min incremented to 23135 V/h on an Ettan™ IPGPhor II™ (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed according to Görg [27] and the GE Healthcare protocol for 13-cm IPG strips pH 3-10 to optimize the running conditions, resulting in a final 13 h 30 min run. Focused IPG strips were then stored in plastic bags at -80°C. Before running the second dimension, strips were incubated twice 15 min in equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer pH 8.8). For the first equilibration 1% DTT and bromophenol blue were added to the equilibration buffer and for the second 4% iodoacetamide and bromophenol blue were added. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied to 12.52% polyacrylamide gels in a Hoefer™ SE 600 Ruby® (Amersham Biosciences) unit. IPG strips were inserted in the stacking gel for SDS-PAGE using a method modified to improve protein resolution [25, 26]. Gels were fixed in 40% methanol/10% acetic acid for 1 h then stained overnight in Coomassie Brilliant Blue G-250 [24]. Coomassie-stained gels were scanned on a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA) and the resulting digitized images were analyzed using Image Master 5.0 software (Amersham Biosciences; GE Healthcare).

2.6. *Protein digestion*

The excess of staining was removed from the gel, and the proteins were located. The spots from 2D-PAGE were excised manually with sterile tips under sterile dust-free environment and transferred to individual tubes for enzymatic digestion. For the protein digestion, the gel pieces were incubated with trypsin at 37°C overnight. Concerning the peptide extraction, the reaction media was acidified with Formic acid (FA) to stop the enzymatic digestion. Then, the supernatant containing the digested peptides was removed to another tube, and additional peptides were extracted from the gel with a 50 % acetonitrile/0.1 % TFA solution. After the extraction step, the supernatant and the extraction solutions were combined and analyzed by MS, or stored frozen for further analysis.

2.7. *Matrix formulation and sample deposition*

Matrix solution was prepared by dissolving 10 mg of α-CHCA in 1mL of H_2O/acetonitrile/TFA (49.95:49.95:0.1). The sample (10 µL) was mixed with 10 µL of the α–CHCA matrix solution with vortexing for 30 s. One microliter of the mixture was spotted on a MALDI-TOF MS target plate and allowed to dry for 5 min.

2.8. *MALDI-TOF MS analysis*

An Ultraflex II MALDI-TOF/TOF-MS instrument from Bruker Daltonics equipped with a 200 Hz Smart beam laser system, was used to obtain peptide mass fingerprints (PMF). MALDI mass spectra were acquired as recommended by the manufacturer. Measurements were taken in reflector positive ion mode in the mass range of 600-3500 Da. Close external calibration was performed with the monoisotopic peaks of the bradykinin (757.3992), angiotensin II (1046.5418), angiotensin I (1296.6848), substance P (1347.7345), bombesin (1619.8223), renin substrate (1758.9326), ACTH clip 1-17 (2093.0862), ACTH 18-39 (2465.1983), and somatostatin 28 (3147.4710). Mass spectrum analysis for each sample was based on the average of 500 laser shots. The peak lists were generated from the mass spectra using the peak detection algorithm SNAP from the FlexAnalysis 3.3 software (Bruker Daltonics). Peptide mass fingerprints were searched with the MASCOT search engines. MASCOT parameters were as follows: variable modifications, oxidation (M); fixed modifications, carbamidomethyl (C); Swiss-Prot database; one missed cleavage allowance, and a peptide tolerance of up to 100 ppm. The default significance threshold, $p < 0.05$, was used. A match was considered successful if the protein identification score was located outside the random region and the protein analysed scored in the first position. Figure 1 summarises the methodology used in the present study.

3. Results

In this study a comparative proteomic analysis was performed between strain C5478 without antimicrobial stress (wild-type) and C5478 stressed with cefotaxime.

A stress or shock involves a change from one environmental condition to another, so the natures of both the original condition and the shock are important in defining the appropriate response. Here, we reasoned that comparing the proteomes of a naturally occurring ESBL-producing *E. coli* strain (C5478 wild type strain) and the ESBL-producing *E.*

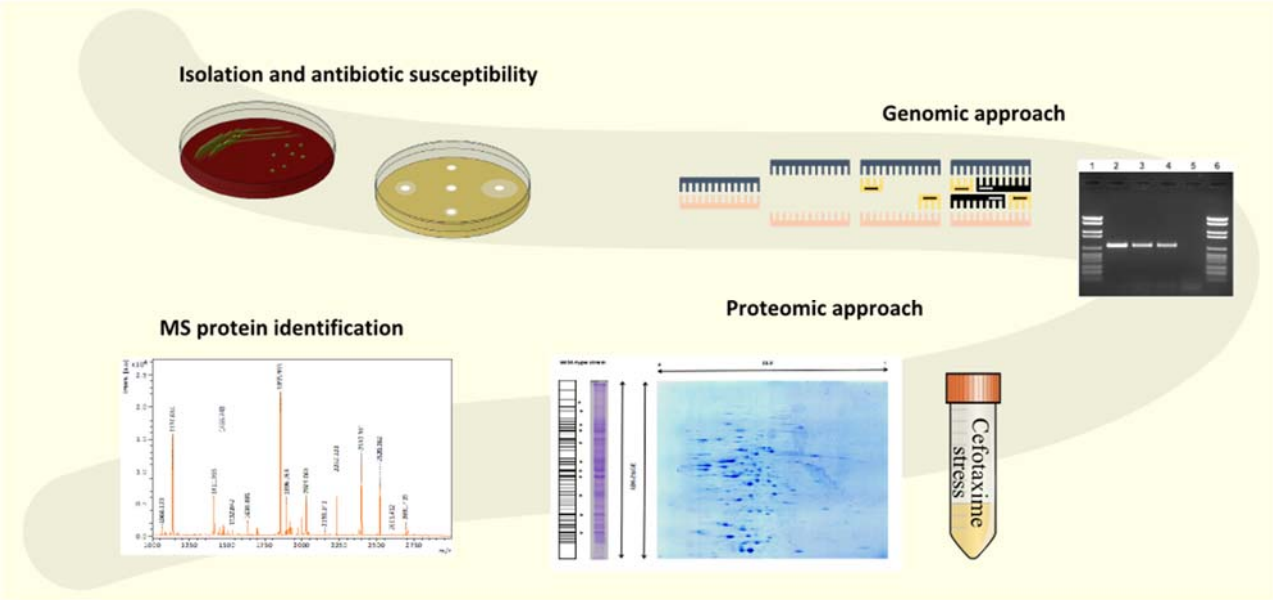

Figure 1. Typical genomic and proteomic workflow representing the classical components of genetical study and protein identification.

coli strain stressed by CTX (stressed strain) would reveal key differences that might be necessary for bacterial colonization in the presence of β-lactams. We used 2D-PAGE and MALDI-TOF MS to initially screen for significant differences in the distribution of proteins between wild-type and stressed strains (Supplementary material, Tables 1 and 2).

The comparative proteomic analysis revealed that the abundance of numerous protein species changed in the strain stressed by CTX compared to the non-stressed wild-type strain. A total of 188 spots were excised from the 2-DE gel of the wild-type strain, 112 of which were successfully identified by MS, representing 110 different proteins (Supplementary material. Table 1). Concerning the 2-DE gel of the CTX-stressed bacteria, 171 spots were excised and 156 were identified, representing 143 different proteins (Supplementary material, Table 2). The proteins identified in both strains were categorized according to their biological functions. These proteins were involved in metabolism, protein synthesis, cell division, stress responses, antimicrobial resistance, transport, and unknown functions among others (Figure 2).

Various proteins were found in more than one spot on the two gels. From the gel of proteins from the wild type ESBL-producing strain, Mdh (spots 74 and 117), Tuf1 (spots 110, 127 and 128), YajQ (spots 36, 56 and 157), GpmA (spots 46 and 50), PflB (spots 169 and 172), YeaD (spots 76 and 80), GadB and GadA (spots 114 and 126), DaaA (spot 32 and 34) were all present in more than one spot. In the gel of proteins from the CTX-stressed bacteria, Tsf (spots 243 and 337), Dps (spots 198 and 200), Pgk (spots 268, 339 and 344), Pnp (spots 303 and 316), rpoB (spots 252 and 315), Gnd (spots 280 and 345), PflB (spots 327 and 328), SlyA (spots 213 and 262), Prs (spots 285 and 360), Tpx (spots 205 and 281), TalA (spots 253 and 254), CysK (spots 253 and 254), MalE (spots 243, 266 and 338), Ssb (spots 207 and 208), AcnA (spots 322 and 323), TktA (329 and 330) and ClpB (spots 312 and 331) were present in more than one spot. Proteins that migrate at more than one distinctive position during electrophoresis have different charges and masses. Hence, these spots could correspond to proteins with different posttranslational covalent modifications [6, 14].

A total of 268 proteins were distinctively expressed in both samples. The proteins that decreased in percentage under CTX stress (Figure 3) were principally proteins involved in transport and ATP-binding (like ArgT, FliY and NmpC), glycolysis and gluconeogenesis (like GapA, GpmA, PykF and OckA), transcription and RNA processing (like DaaA, RopB, Fur and Pnp), translation (like RpsA and RpsB), proteolysis (PepQ and PrlC), fatty acid biosynthesis (like AccC and FabF), and cell shape (DdlB).

In contrast, the percentage of stress response proteins expressed was much greater in bacteria under CTX stress (Figure 4) such as those involved in mechanisms of acid resistance (GadA, GadB, WrbA), oxidative stress (like Tpx, SodB and OsmC), other general cellular stress responses involving cold and heat shock proteins (DnaK, ClpB, ClpP). Among the numerous changes noted in the stressed strain, the percentage of proteins related to DNA damage, cell division, cell redox, innate immune response (like FtsZ, FtsY, SsB and YbbN), starvation (Dps) and antimicrobial resistance (OmpX, GyrB, FabI) increased. In the stressed bacteria, there were also increased percentages of enzymes involved in protein biosynthesis (like AspS, FusA and GlyS), the tricarboxylic acid cycle (like MdH, SucB and SucC), amino-acid biosynthesis (like CysK and CarB), nucleotide and purine biosynthesis (PurA and Prs), the pentose shunt (like TalA, TalB and GnD) and transketolase activity (TktA) among others.

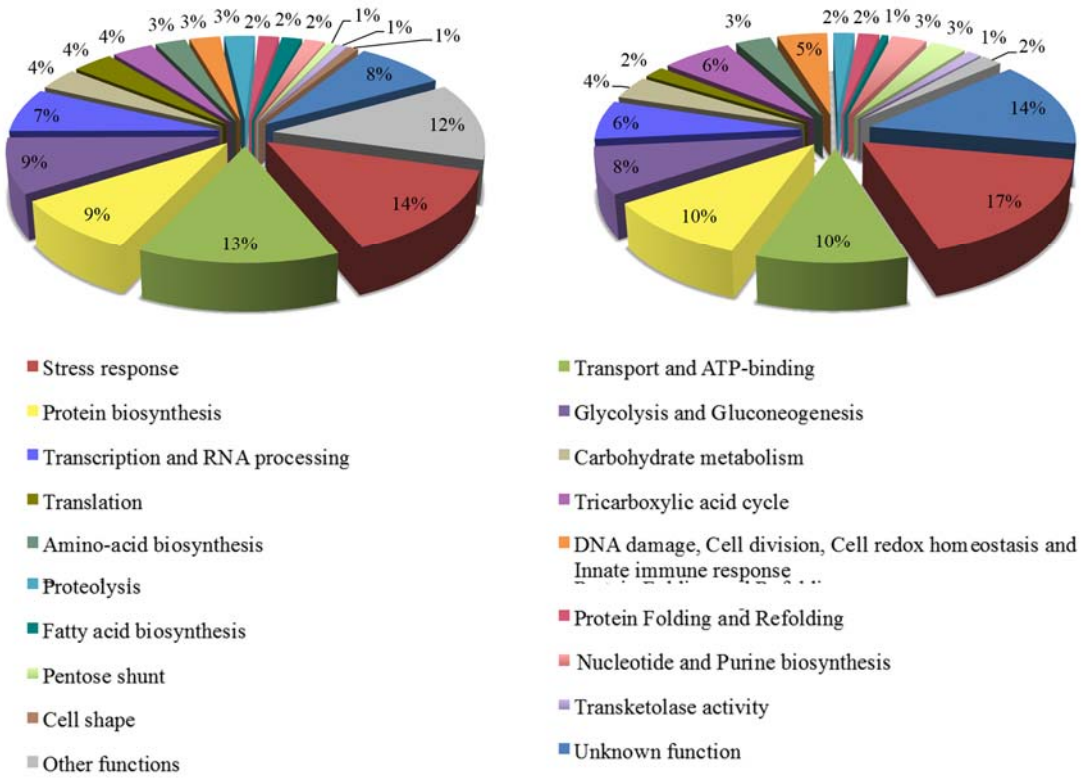

Figure 2. Distribution of biological functions of proteins expressed in wild type (left) and CTX stressed *E. coli* C5478 (right)

4. Discussion

The widespread use of antimicrobials to treat infections means that cases of bacterial resistance to these drugs are becoming more frequent, presenting a continuing challenge in medicine. Multidrug resistance is a main cause of clinical failure in treating bacterial infections. Increasing evidence suggests that bacteria can resist multiple antimicrobials through intrinsic mechanisms that rely on gene products such as efflux pumps that expel antimicrobials and specific membrane proteins that block the penetration of drug molecules.

The response of bacterial metabolism to environmental perturbations is characterized by a fast and appropriate adjusting of physiology on every level of the cellular and molecular network. The entire sequence of the *E. coli* genome has been available since 1997 greatly facilitating proteomic methodologies of 2-DE with MALDI-TOF MS analysis of proteins related to antimicrobial resistance of this bacterium.

In CTX-stressed bacteria, there were mainly decreases in percentages of proteins involved in transport/ATP-binding, glycolysis, transcription/RNA processing, translation, proteolysis, fatty acid biosynthesis and also some proteins related to oxidative and acid stress responses. Though enzyme analyses and other physiological data are still required, we can make some tentative conclusions about these overall changes in bacterial physiology. The consequences of decreased protection against oxidative stress and acid stress may have been compensated for by increased levels of proteins active in DNA damage repair, cell division, cell redox homeostasis, the innate immune response as well as proteins associated with antimicrobial resistance, tellurium resistance, general/heat stress and starvation. The stressed bacteria had acquired defence mechanisms normally related to the stress response, and an increased tolerance to antimicrobials was confirmed. Increases in the percentages of proteins involved in the stress response are often at the expense of proteins implicated in translation. Similarly we found that the increase in tolerance to cefotaxime in the stressed strain has deleterious effects on ribosomal activity.

These metabolic modifications could indicate the biological cost of antimicrobial resistance, though it is possible that other changes in these proteins that were not determined on these 2-DE gels could explain the results. Any such costs are likely to be mitigated by subsequent evolution as described by [29].

4.1. *Stress response*

Stress responses are usually a combination of specific responses aimed at minimizing deleterious effects (e.g. catalase production during oxidative stress) or repairing damage (e.g. chaperone expression under temperature stress) and general responses.

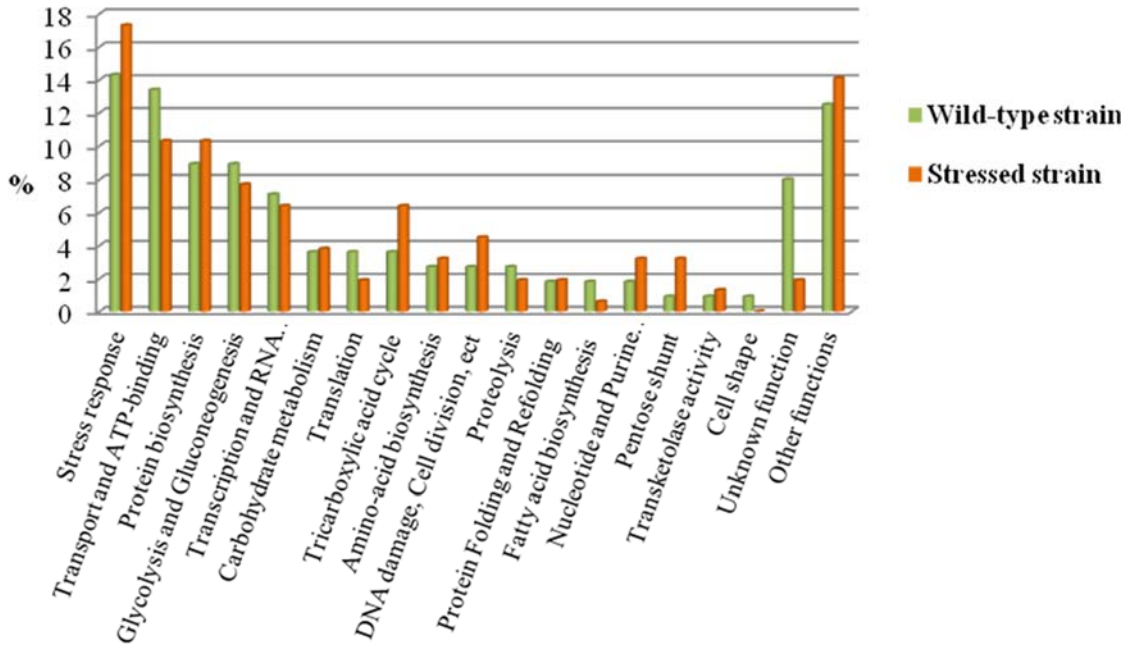

Figure 3. Percentage distribution by biological function of proteins identified in the wild type and CTX stressed *E.coli* C5478 strains

The results of this study revealed a significant increase in the percentage of stress response proteins, like GadA and GadB, present in the naturally occurring and stressed bacteria. GadA and GadB are the most efficient acid resistance system [30]. Defences against oxidative stress involve constitutive and tightly regulated adaptive mechanisms to prevent and scavenge oxidants as well as to repair damaged biomolecules. The widely accepted simplified paradigm of defence against oxidative stress is that superoxide is removed by superoxide dismutases such as SodB (spots 26 and 209) generating hydrogen peroxide, which is removed by catalases, like KatE (spots 174 and 324) and peroxidases like AhpC (spot 212, MW 20862 and PI 5.03). Several of these defences are controlled by regulators that respond to iron such as Fur (spot 2, MW 17012 and PI 5.68) or oxygen tension such as ArcA (spot 52) [31].

In the CTX-stressed bacteria, besides the ribosome-associated Trigger factor Tig (spot 284), the cytoplasm of the strain contains two of the three FK506-binding proteins (FKBPs) SlyD (spot 240) and FklB (spot 215). FKBPs are enzymes grouped into a superfamily of peptidyl-prolyl-cis/trans-isomerases (PPIases) that act as chaperones in folding proteins. Many proteins involved in disease processes require modification, thus PPIases can play an important role

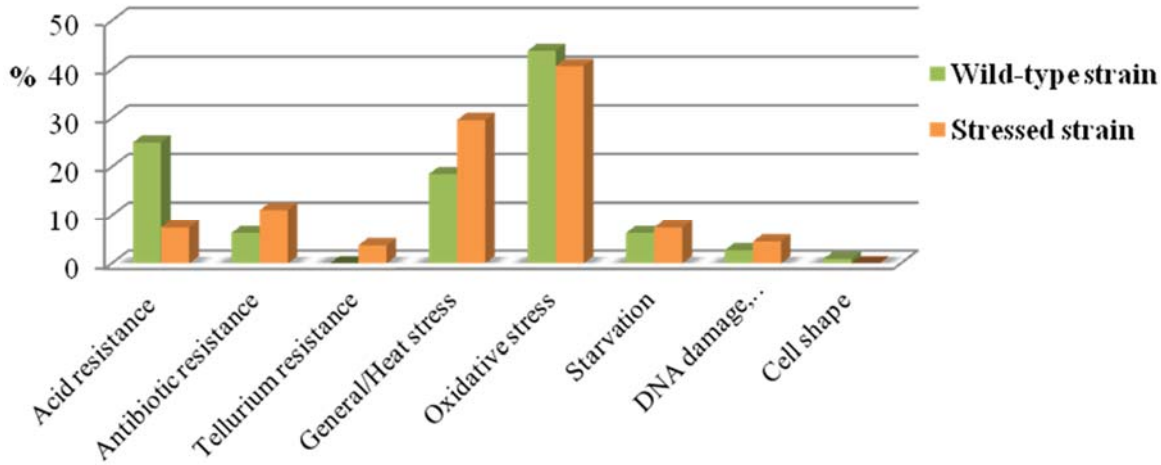

Figure 4. Percentage distribution of stress response proteins identified in wild type and CTX stressed *E. coli* C5478 strains

in pathogenesis [31].

Other proteins related to oxidative stress defence in the stressed strain were identified here such as CysQ (spot 219), YdhR (spot 323), YhfA (spot 191), MaeB (spot 310) and Tpx (spots 205 and 281). The osmotically inducible protein OsmC, which was identified in both strains (spots 7 and 190), is involved in hydroperoxide protection particularly in host–pathogen interactions [32].

Some heat shock proteins (HSPs) function as molecular chaperones or have functions linked with DNA replication, cell division, and maintenance of active protein conformations [33]. The number of proteins related to heat stress increased under CTX stress, for example ClpB (spots 312 and 331) and HtpG (spot 293), which participate in protein folding in stressed *E. coli* cells [34].

KlaB (TelA, MW 42130 and PI 6.05) was one of the stress response proteins identified in the stressed strain (spot 287). KlaB is responsible for tellurium resistance (Supplementary material, Tables 1 and 2). Tellurite is toxic to *E. coli* through an unknown mechanism that involves an increase in oxidative stress. However recently, biochemical, genetic, enzymatic and molecular approaches were used to demonstrate, for the first time, that tellurite toxicity in *E. coli* involves superoxide formation. Altogether, this study strongly indicate that the dramatic toxic effect of potassium tellurite in *E. coli* can be associated with the chemical activity of the tellurium oxyanion at various levels of bacterial metabolic pathways including, among others, inactivation of [Fe-S] center-containing dehydratases, cytoplasmic thiol oxidation, enzyme and protein carbonylation and membrane peroxidation [35].

4.2. *DNA damage, cell division, cell redox homeostasis and the innate immune response*

In the stressed strain derived from C5478, there were increased percentages of proteins related to DNA damage, cell division and cell redox homeostasis. Of the cell division proteins, FtsZ found in the stressed strain (spot 261) is of great interest as it is the earliest actor in the division pathway and its level dictates the frequency of division [36]. Furthermore, FtsY, also identified in the stressed strain (spot 314, MW 54480 and PI 4.46), is a signal recognition particle receptor in *E. coli* that mediates the targeting of integral membrane proteins to translocons by interacting with both signal recognition particle (SRP)-nascent polypeptide–ribosome complexes and the cytoplasmic membrane [37].

The SsB protein, one of the proteins in the DNA damage category, was found in the stressed strain in two spots (207 and 208). SsB protects and sequesters single-stranded DNA regions until the double helix can be reformed [38].

The relative amounts of cell wall proteins found in the stressed bacteria are quite high. Constant turnover of the cell wall provides ample decoy material for *E. coli* to engage host innate defences and the rate of cell wall production is directly proportional to the rate of colonization of the human tract [39]. Penicillin-binding proteins (PBPs), the target enzymes of β-lactam antimicrobials such as cefotaxime, play an essential role in bacterial cell elongation, cell division and cell wall biosynthesis. PBPs are membrane-bound enzymes that are widespread in *E. coli* [40].

The SOS response supports bacterial propagation by inhibiting cell division during repair of DNA damage and cell survival. The extent of induction of the SOS response can be determined by measuring β-galactosidase synthesis from a LacZ (spot 319, MW 117300 and PI 5.20) fusion with the SOS-regulated promoter of the *sfi*A gene, which prevents FtsZ (spot 261, MW 40299 and PI 4.63) polymerization and inhibits cell division when SOS is activated [37]. The immediate response of bacterial pathogens to antimicrobial therapy is of key interest when considering how to deal with infectious diseases.

The FtsZ protein, identified in the stressed bacteria, is crucial to the cell-division process. It assembles as a dynamic ring on the inner surface of the cytoplasmic membrane at the place where division will happen, with the formation of the ring being the signal for septation to begin [36].

It is important to also point out the presence of the FtsY protein in the stressed bacteria. The capability of transformants producing chimeric FtsY to process β-lactamase was evaluated in a recent study [37]. While β-lactamase is not dependent on signal recognition particle (SRP) for export, it does require SRP function for cleavage of the signal peptide, as leader peptidase is an SRP-dependent membrane protein in *E. coli* [41].

4.3. *Antimicrobial resistance*

More of the antimicrobial resistant proteins Ompx, GyrB and FabI were observed in the stressed strain. OmpX is present in the both strains (spot 14, MW 67648 and PI 5.30) and (spot 196, MW 18648 and PI 5.30). Because of the function of this integral outer membrane protein in membrane permeability, OmpX may be involved in controlling the penetration of antimicrobials such as β-lactams and fluoroquinolones through the enterobacterial outer membrane as is known for chloramphenicol, tetracycline, and kanamycin [42]. OmpX belongs to a family of highly conserved bacterial proteins that have been assigned key functions in promoting bacterial adhesion and entry into mammalian cells [43].

Usually, resistance to fluoroquinolones arises spontaneously because of point mutations that result in amino acid substitutions within the topoisomerase subunits GyrA, GyrB, ParC or ParE, a decreased expression of outer membrane porins, or an over expression of multidrug efflux pumps [44]. GyrB was identified (spot 325, MW 90179 and PI 5.72) in this study, which is consistent with the quinolone acid resistance observed in the strain. The wild-type strain was known to have two amino acid changes in GyrA (S83L+D87G) and one in ParC (S80I) responsible for ciprofloxacin and nalidixic acid resistance [23].

The presence of the protein FabI (enoyl reductase), an im-

portant enzyme in fatty acid biosynthesis, was observed in the stressed bacteria (spot 245, MW 28074 and PI 5.58). Triclosan, which has been described as a broad-spectrum antimicrobial and is found in many antibacterial consumer products, is now known to specifically target FabI in *E. coli* [45].

The likely loss of energy production in the stressed strain caused by the decreased percentage of proteins involved in the glycolytic pathway may be a cost that resistance imposes on the fitness of bacteria [14]. Besides glycolysis, other pathways such as the tricarboxylic acid (TCA) cycle are likely to be involved in regulating and responding to CTX bactericidal effects, which may explain the increase in the amount of TCA cycle related proteins. Further study of the relationships between iron, metabolism, and iron-sulfur cluster-containing proteins [46] is necessary to identify any post-transcriptional events not captured in gene expression studies that trigger this common cell death pathway [47].

4.4. *Transport proteins*

The wild-type strain is multidrug resistant. Both the wild-type strain and the stressed strain can resist antimicrobial agents by active efflux of the agents using translocation machinery [48]. This is borne out by our results showing that some of the most abundant proteins, more than 13% and 10% of those identified in the non-stressed and stressed conditions respectively, were linked to transport and ATP-binding functions (Figure 2). These included three proteins related to ATP-binding detected in the stressed strain, Acs (spot 329), GlnA (spot 295) and PpsA (spot 304). The ATP synthase subunit alpha (AtpA) and beta (AtpD) were present in the wild-type and stressed strain. In *E. coli* ATP synthase activity provides proton motive force for efflux pump activity [49]. Additionally, the SecD protein identified in the stressed strain (spot 202, MW 66648 and PI 8.62) is a transport protein implicated in bacterial pathogenesis and in the secretion of virulence proteins [39].

Antimicrobial resistance often entails a metabolic cost because the resistance mutations typically occur in genes of target molecules that have essential functions in the cell. In fact, mutation is a primary cause of bacterial resistance to antimicrobials. In *E. coli* active efflux systems are common mechanisms of reduced susceptibility to fluoroquinolones that may confer resistance, particularly when associated with mutation in GyrA, GyrB or ParC [49] like those in GyrA and ParC in the wild-type C5478 strain studied here.

As previously mentioned the enzyme FabI was identified in the stressed bacteria. Given the multi-drug efflux pumps in *E. coli*, over expression of one of the pumps could participate in resistance mediated by a FabI mutation, which might have the effect of spreading antimicrobials.

4.5. Amino acid and protein biosynthesis/metabolism

The number of proteins related to amino acids, protein biosynthesis and metabolism increased in the stressed bacteria. One example is the CysK protein (spots 253 and 254) that has elsewhere been shown to mediate tellurite resistance in *E. coli*. Pyridoxine 5'-phosphate synthase (PdxJ, spot 235) was present in the stressed bacteria. The genes *pdxJ* and *pdx*A encode proteins involved in vitamin B_6 biosynthesis. It was proposed that PdxJ enzymes could be ideal therapeutic targets against bacterial pathogens [50].

Transaldolase A (TalA), found in the stressed bacteria (spot 253 and 254), is involved in the nonoxidative branch of the pentose phosphate pathway. The gene coding for the isozyme TktA is also detected in the stressed strain (spots 229 and 230) and is known to be upregulated by stress conditions [51].

The Upp protein was found in the stressed bacteria (spot 214). It was shown that energy production genes such as SucB are involved in persister survival and tolerance of multiple antimicrobials and stresses in *E. coli* [52]. Upp, expressed naturally in *E. coli*, converts the chemotherapeutic drug 5-fluorouracil (5-FU) into 5-fluorouridine monophosphate, an extremely toxic compound. Adenoviral delivery of the Upp gene to human cancer cell lines increased their sensitivity to 5-FU [53]. The *E. coli* enzymes DeoD (spots 33, 217) have also been used in gene therapy studies [53].

The main HSPs DnaK, DnaJ, GroEL are molecular chaperones that assist in the correct folding and assembly of proteins and are implicated in various cellular processes including DNA replication, RNA transcription, flagella synthesis and UV mutagenesis [54]. HSPs participate in the immune response to bacterial infections and the development of autoimmune diseases [55]. Different classes of HSPs from different bacteria can directly induce cytokine expression and secretion in macrophages [56]. We found several chaperones related to the stress response were found in the both strains but they were more abundant in the stressed strain. It has been demonstrated previously that β-lactamase interacts with GroEL and GroES [57, 58] and that export of β-lactamase is defective in GroEL and GroES mutants [41].

Glycopeptides such as vancomycin and teicoplanin target the peptidoglycan cell wall of bacteria by selectively binding to the D-alanyl–D-alanine termini of peptidoglycan precursors preventing cross linking to adjacent strands. The presence of DdlB, an attractive target for developing novel antimicrobials [59], was detected in the wild-type strain.

5. Conclusion

In the present study the use of 2D-MALDI-TOF MS provided wide proteome coverage as demonstrated by the number of proteins identified (112 in the wild-type strain and 156 in the stressed strain). This made it possible to more comprehensively evaluate changes in protein expression in response to selection with CTX. The numerous changes in protein levels observed in the stressed strain suggest that resistant micro-organisms may adapt to adverse environmental conditions [60, 61] with implications for several aspects of bacterial metabolism, which may be mirrored in their virulence parameters.

The ability of resistant bacteria to survive in a population and in the community depends on numerous factors, such as biological fitness. In this case, antimicrobial resistance can be stabilized with fitness-restoring compensatory mutations and may permit completely resistant strains to compete successfully with susceptible strains in an antimicrobial-free environment [29]. In conclusion, we have demonstrated that a wide range of proteins change in abundance in a diverse secondary response to treatment with cefotaxime, a third-generation cephalosporin. For the proteins already associated with antimicrobial resistance, such as OmpX, FtsZ and FtsY, the modifications are likely due to the immediate effects of encountering CTX. These findings will be helpful for understanding more generally what constitutes a functional proteome and, in particular, how antimicrobial-resistant mechanisms work.

6. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/140/0

Table 1- Protein identification by MALDI-TOF/MS of 2-DE gel spots from wild-type strain ESBL-*E. coli* C5478.

Table 2 - Protein spots identification by MALDI-TOF/MS of 2-DE gel spots from stressed strain ESBL-E. coli C5478.

Acknowledgments

Hajer Radhouani, Júlio D. Nunes-Miranda, Hugo M. Santos and Luís Pinto were supported by grants SFRH/BD/60846/2009, SFRH/BD/80496/2011, SFRH/BPD/73997/2010 and SFRH/BD/81307/2011, respectively, and by FCT (Fundação para a Ciência e a Tecnologia) and POPH/FSE (Programa Operacional Potencial Humano/Fundo Social Europeu).

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